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Pathways of complement activation in glomerulonephritis

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Pathways of complement activation in glomerulonephritis. Systemic lupus erythematosus and hypocomplementemic glomerulonephritis are characterized by depressed CH_{50} and C3 levels, but the complement profiles in these two diseases show marked differences. Measurement of hemolytic activity of C1, C4 and C2 and of protein concentrations by radial immunodiffusion of C1q, C1s, C1 inhibitor, C4 and C3 again showed depression of all complement components in systemic lupus erythematosus as in experimental immune complex disease but selective lowering of CH_{50} and C3 in hypocomplementemic glomerulonephritis. Suppressive therapy in systemic lupus erythematosus was accompanied by clinical improvement and a rise of the complement component levels to normal whereas depressed complement and C3 levels persisted after similar courses of therapy and during symptomatic remissions of hypocomplementemic glomerulonephritis. The ratio of C1 inhibitor/C1 was elevated in systemic lupus erythematosus and normal in hypocomplementemic glomerulonephritis.

The return of complement components to normal with immunosuppressive therapy and the elevation of the C1 inhibitor/C1 ratio in systemic lupus erythematosus are in accord with the activation of complement by immune complexes. In hypocomplementemic glomerulonephritis the persistence of low levels of complement with immunosuppressive therapy and the normal C1 inhibitor/C1 ratio suggest that the activation of complement in this disease is not by immune complexes and the conventional pathway.

One child with hypocomplementemic glomerulonephritis had an unusual C2-deficiency, probably on a congenital basis, in addition to the depression of CH_{50} and C3 typical of this disease. The possible relationship of congenital complement abnormalities to the development of glomerulonephritis deserves further consideration.

Cheminement des réactions de l'activation du complement dans la glomérulonéphrite. Le lupus érythémateux disséminé et la glomérulonéphrite avec hypocomplémentémie se caractérisent par un abaissement des taux de CH_{50} et de C3, bien que les profils du complément présentent une notable différence dans ces deux maladies. La mesure de l'activité hémolytique de C1, C4 et C2 ainsi que la mesure des concentrations protéiniques par immunodiffusion radiaire de C1q, de C1s, de l'inhibiteur C1, de C4 et de C3 montrent un abaissement de toutes les composantes du complément dans le lupus érythémateux dissiminé, comme d'ailleurs dans les maladies immunologiques expérimentales. Cependant, une diminution sélective de CH_{50} et de C3 fut observée dans la glomérulonéphrite avec hypocomplémentémie. Le traitement à l'aide d'immunosuppresseurs dans le lupus érythémateux dissiminé s'est traduit par une amélioration clinique et une normalisation des composantes du complément. Par contre, la diminution des taux de complément et de C3 persista durant le traitement par immunosuppresseurs et durant les rémissions symptomatiques de la glomérulonéphrite avec hypocomplémentémie. Le rapport de l'inhibiteur C1/C1 était élevé dans le lupus érythémateux dissiminé, mais normal dans la glomérulonéphrite avec hypocomplémentémie.

La normalisation des composantes du complément lors du traitement par immunosuppresseurs ainsi que l'élévation du rapport inhibiteur C1/C1 dans le lupus érythémateux dissiminé correspondent à une activation du complément par des complexes immuns. La persistance de taux abaissés du complément et le rapport normal de l'inhibiteur C1/C1 notés au cours du traitement par immunosuppresseurs dans la glomérulonéphrite avec hypocomplémentémie suggèrent que l'activation du complément dans cette maladie ne dépend pas de complexes immuns et ne suit pas le cheminement normal des réactions d'activation du complément.

Nous avons noté qu'un enfant atteint de glomérulonéphrite avec hypocomplémentémie présentait une déficience inhabituelle en C2, anomalie probablement d'origine congénitale, en plus de l'abaissement de CH_{50} et de C3 qui sont observés de façon caractéristique dans cette maladie. La possibilité qu'il existe une relation entre les anomalies congénitales du complément et l'apparition d'une glomérulonéphrite mérite de retenir l'attention.

Depression of serum complement and the deposition of complement and immune globulin on glomerular basement membrane has been interpreted as evidence of an immune etiology of glomerulonephritis [1, 2, 3]. Activation of the complement cascade by antibody to glomerular basement membrane or by immune complexes leads to the release of biologically active fragments from C3 and C5 which induce local cellular injury [4]. Experimentally, the activation of complement by antigen-antibody reactions, both *in vivo* and *in vitro*, results in consumption of each of the 11

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complement proteins in sequence from C1q through C9 [5]. Of human diseases with low serum complement levels, the profile in systemic lupus erythematosus shows depression of C1–9 as expected in an immune complex disease. The profile in hypocomplementemic glomerulonephritis [6, 7] shows sparing of the early complement components and depression of C3 through C9 [5, 8]. The pattern suggests that activation of complement in this disease is not through the classical pathway after the binding of C1q to antibody, but by a recently described alternate pathway of complement activation through a serum beta-globulin, C3 proactivator [9].

Serial complement profiles in children with systemic lupus erythematosus and hypocomplementemic glomerulonephritis were compared for evidence of complement activation by the classical pathway or through C3 proactivator and the second pathway. Measurements were made by hemolytic assay for functional activity, and by radial immunodiffusion for the protein concentration of the complement components. In systemic lupus erythematosus, the rise in component titers to normal with suppressive therapy and the increase in the ratio of C1 inhibitor to C1s (evidence of immune complex activation of C1) suggests that complement is utilized in the classical pathway. In hypocomplementemic glomerulonephritis, persistent depression of C3 and CH_{50} during suppressive therapy and the normal ratio of C1 inhibitor to C1s support the thesis that C is consumed in the alternate pathway. One child with hypocomplementemic glomerulonephritis showed marked depression of C2 as well as C3 but normal or elevated levels of C1 and C4. Levels of C2 were not as low as those seen in hereditary C2-deficiency [10], but the presence of decreased C2 in a healthy sibling indicated that the deficiency was probably not related to consumption.

Clinical Material

Serial studies were done on sera from four females, ages ten to fourteen, with systemic lupus erythematosus and three females, ages six to twelve years, with hypocomplementemic glomerulonephritis. Two of the four with systemic lupus erythematosus, Cases 1 and 2, were studied within three weeks of the onset of symptoms and they were followed during the initial period of steroid therapy. Fever, rash and arthralgias preceded evidence of biopsy-proven glomerulonephritis. Case 3 was first examined during an exacerbation of fever, arthritis and carditis for which she had been treated sporadically for three years. Case 4 had been treated for central nervous system disease and glomerulonephritis with a variety of immunosuppressive agents for three years, and she was asymptomatic when first evaluated.

The three females with hypocomplementemic glomerulonephritis were asymptomatic during the period of observation. Minimal proteinuria or microscopic hematuria were the only signs of renal disease. Two children (Cases 5 and 6) with membranoproliferative glomerulonephritis on renal biopsy had completed courses of therapy with combined cyclophosphamide and prednisolone when first tested. Prednisolone was tapered during the period of observation. Case 7 had proliferative glomerulonephritis on initial biopsy and mild membranoproliferative glomerulonephritis on repeat biopsy one year later. Her only treatment was a two week course of prednisone and heparin three months before referral. Control children were patients on the pediatric wards and in the renal clinic of New York Hospital, and they represented a variety of diseases such as idiopathic nephrosis, familial nephritis, healed acute glomerulonephritis and recurrent hematuria.

Methods

Fresh blood specimens were allowed to clot at room temperature for one hour or less and the serum was stored at $-70°$ C until used. Sheep erythrocytes collected in Alsevers solution were washed in EDTA buffer, 0.01 M, pH 7.4, and reacted with commercial sheep hemolysin, 1/1500 (Baltimore Biological Laboratories, Baltimore, Maryland). Functionally pure C2 was prepared by the method of Nelson et al [10]. Buffers used were: 1) veronal-buffered saline (0.15Γ/2, pH 7.5) with 0.1% gelatin, calcium 1.5×10^{-4} M, and magnesium 1.0×10^{-3} M; 2) veronal-buffered saline (0.075 Γ/2, pH 7.5) containing dextrose 2.5%, gelatin 0.1%, calcium 1.5×10^{-4} M, and magnesium 0.5×10^{-4} M (designated DGVB); 3) EDTA buffer, 0.01 M, was prepared by diluting stock EDTA (pH 7.5, 0.10 M), and it contained 0.1% gelatin. Hemolytic complement titers (CH_{50}) were measured by the micro-method of Kent and Fife [11]. The normal range by this method is 150 to 250 units. C1 titrations were done by a modification of the method of Borsos and Rapp [12]. After reacting the erythrocyte-intermediate $EAC4_{gp}$ with serum dilutions to form $EAC1_{hu}4_{gp}$, the cells were washed with DGVB and then incubated two hours at 30° C to allow complete activation of C1. Then the $EAC1_{hu}4_{gp}$ were reacted with functionally

pure C2 and guinea pig serum in EDTA buffer in excess as in the original method. C4 assays were performed by the method of Ruddy and Austen [13], and C2 titers were measured by the method of Ruddy et al [14]. Radial immunodiffusion measurements of serum protein concentrations of C1q, C1s, C1-inhibitor, C3 and C4 were done by the method of Mancini, Carbonara, and Heremans [15]. Antisera were prepared by injecting rabbits or goats with purified preparations of C1q [16] and C1s [17]. Antisera against C1-inhibitor and C4 were prepared by injecting specific antigen-antibody precipitates into the same animal species donating the antibody [18].[1] Anti-C3 was prepared by the method of Mardiney and Müller-Eberhard [19]. Concentrations of C1q and C4 were expressed as μg/ml, C3 as mg/100 ml, and C1s and C1-inhibitor as percent of a normal sera pool.

[1] Antisera against C1-inhibitor and C4 were kindly provided by Drs. Fred Rosen and Peter Schur, respectively.

The effect of immune complexes on complement components and C1-inhibitor in normal serum was studied *in vitro*. One milliliter of normal human serum was added to washed preformed BSA-anti-BSA complexes prepared at equivalence containing approximately 1.0 mg of antibody protein. Residual C1q, C1s, C3, and C1-inhibitor protein concentrations were measured in the supernatant after 60 min at 37° C.

Anti-DNA antibodies were demonstrated by the Farr technique [20]. Tests for an inhibitor or inactivator of C2 or C3 in the serum of the C2-deficient child (Case 7) were done by observing the effect of her serum on normal serum titers. To test for C2-inactivation, the C2-deficient serum was incubated with an equal volume of normal serum for 30 min at 30° C, and the titer was compared with a normal control that had been incubated with buffer. Test for C3-inactivation was made by comparing the C3–9 hemolytic activity of guinea pig serum, normal human serum and

Table 1

Hemolytic Assay of complement components in normal adults, control children and children with systemic lupus erythematosus and hypocomplementemic glomerulonephritis

	CH_{50} Units	C1 SFU	C4 SFU	C2 SFU
Normal adults				
No.	9	6	9	6
Range	156–226	58,000–300,00	110,000–290,000	1208–2060
Mean	180	220,000	214,000	1330
±SD	±26	±87,500	±78,000	±332
Control children				
No.	23	9	13	10
Range	137–284	170,000–230,000	57,000–260,000	830–2025
Mean	226	198,000	133,000	1590
±SD	±15.4	±44,000	±54,000	±399
SLE[a]				
1	50	800	490	64
2	50	30,000	490	47
3	109	79,000	32,000	890
4	164	200,000	210,000	1110
HGN[a]				
1	133	109,000	140,000	945
2	95	170,000	166,000	954
3	70	180,000	160,000	42

[a] SLE = systemic lupus erythematosus; HGN = hypocomplementemic glomerulonephritis. Titers represented are those observed initially.

normal human serum plus the C2-deficient serum after incubation at 37° C for 30 min.

Kidney biopsies (done on all patients with the exception of Case 4) were processed and examined in the Department of Pathology, Cornell University Medical College. Fluorescent antibody studies were done with labeled rabbit antiserum to IgG, beta-1C-globulin and fibrinogen. Rabbit antiserum to properdin, kindly provided by Dr. John Boyer, was reacted with the histological section from Case 7 and subsequently with fluorescein-labeled antibody to rabbit gamma globulin.

Results

Complement profile. Complement profiles on single specimens from normal adults, control children and the children with systemic lupus erythematosus and hypocomplementemic glomerulonephritis confirmed previous reports of uniform depression of complement components in active systemic lupus erythematosus, and of selective depression of C3 and terminal components [5, 8] in hypocomplementemic glomerulonephritis (Table 1). Only one child with lupus (Case 4), who was completely asymptomatic after treatment, had normal values for CH_{50} and for complement components. An unexpected finding was the marked C2-deficiency in a child with hypocomplementemic glomerulonephritis (Case 7). The higher values for CH_{50} and components in the control children compared to normal adults probably reflects a non-specific response to inflammation in these hospitalized or clinic patients.

Serial determinations of the complement profile in patients with systemic lupus erythematosus during initial therapy with prednisolone showed significant rises in CH_{50} and in C1, C4 and C2 hemolytic titers (Fig. 1). As steroid therapy was changed to an alternate day schedule or tapered, the titers gradually fell. Serum protein concentrations of C1q, C1s, C4 and C3 similarly rose to normal levels during therapy (Table 2). Decreased ability of serum to bind DNA by the Farr

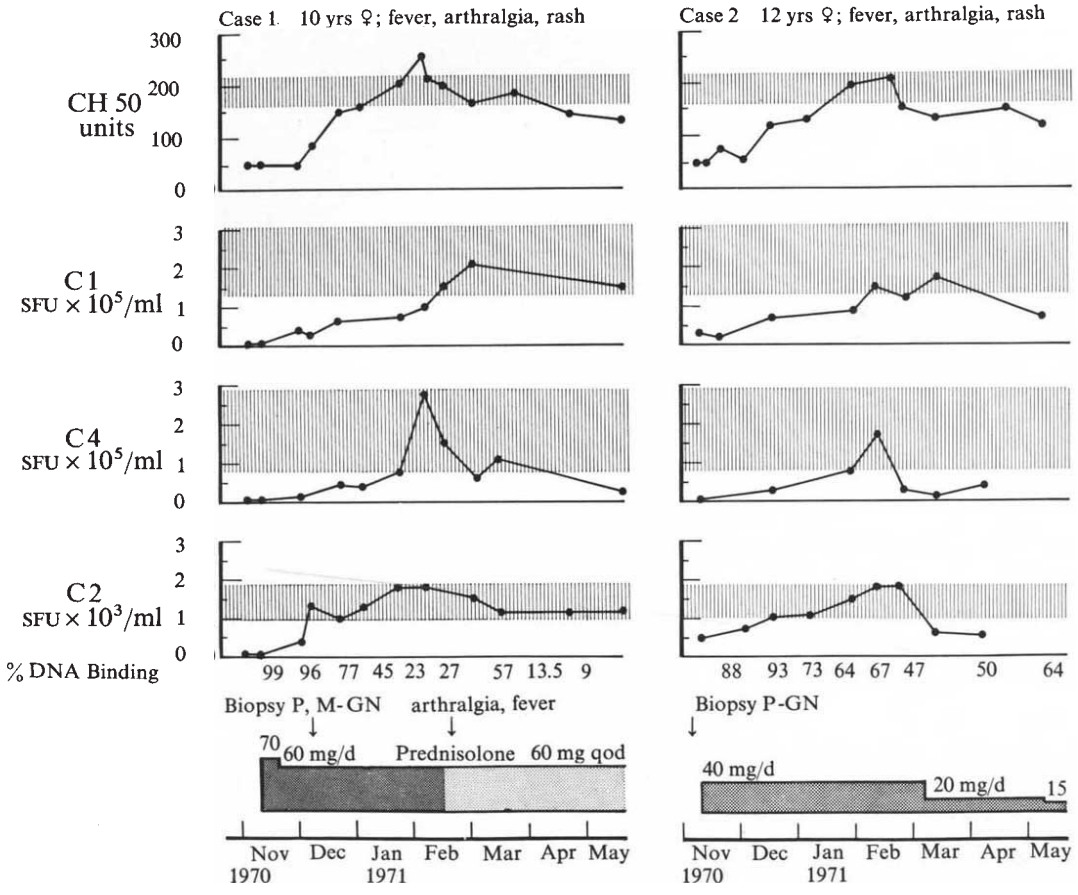

Fig. 1. Hemolytic titrations of whole complement (CH_{50}), C1, C4 and C2 and measurements of anti-DNA antibodies in two patients with systemic lupus erythematosus. The shaded zones indicate the range of C values in control sera.

Table 2

Serum protein concentration in systemic lupus erythematosus and hypocomplementemie glomerulonephritis

Pt.	Date	Clq *μg/ml*	Cls % Normal	Cl-Inh % Normal	C4 *μg/ml*	C3 *mg/100 ml*
Normal[a]		150–210	72–110	100	390–654	105–148
SLE						
1	11/6/70	<18	22.5	55	35	29
	12/9/70	101	81	98	135	74
	4/22/71	165	134	100	270	125
2	11/9/70	67	36	108	175	88
	2/11/71	145	58	200	416	170
	5/6/71	103	64	97.5	242	100
3	10/1/70	55	80	250	263	73
	1/19/71	120	89	155	—	90
4	2/23/71	185	100	155	400	165
	4/27/71	185	92	155	—	172
HGN						
5	9/3/70	150	86	146	603	60
	4/1/71	220	172			43
6	9/3/70	130	120	86	362	50
	2/16/71	140	70	—	—	35
7	9/25/70	230	164	146	624	42
	2/11/70	230	133	—	—	10

[a] Range in 34 normal adults.

test was noted concurrent with the rise in complement. In another child (Case 3) an increase in the dosage of prednisolone sufficient to control symptoms resulted in a rise in the titers and protein concentrations of complement components. The initial complement profile was normal in the child who received prolonged antimetabolite therapy, and it remained normal in the absence of therapy. All children were asymptomatic when their complement profiles were normal.

On the other hand, in hypocomplementemic glomerulonephritis, serum CH_{50} and C3 concentrations remained abnormally low during completely asymptomatic periods (Fig. 2). Two patients were completing prolonged courses of therapy with prednisolone or combined prednisolone and cyclophosphamide (Cases 5 and 6) at the time of initial testing. In addition to low CH_{50} and C3 levels, the third patient (Case 7) showed depressed C2 levels in every specimen. Hemolytic titers for C1 and C4 and the protein concentration of the early components measured by radial immunodiffusion were repeatedly normal in hypocomplementemic glomerulonephritis. Tests for inhibition of C2 and C3 activity in the C2-deficient serum showed no evidence of inactivation of either component.

Specific activity of C1 and the C1-inhibitor/C1 ratio. In systemic lupus erythematosus, depression of the serum protein levels for the C1-subunits, C1q and C1s, was not as marked as for the hemolytic activity of C1. Thus, the functional activity of C1, the specific activity, was diminished as compared to normal serum. In hypocomplementemic glomerulonephritis, the absolute value for C1 hemolytic activity, the protein concentrations of C1q and C1s and the specific activity were normal (Table 2 and 3).

The absolute values for C1-inhibitor were low, normal, or increased during the active phase of systemic lupus erythematosus but the ratio of inhibitor to C1 was elevated in all patients with systemic lupus erythematosus. Normal ratios were found in the children with hypocomplementemic glomerulonephritis.

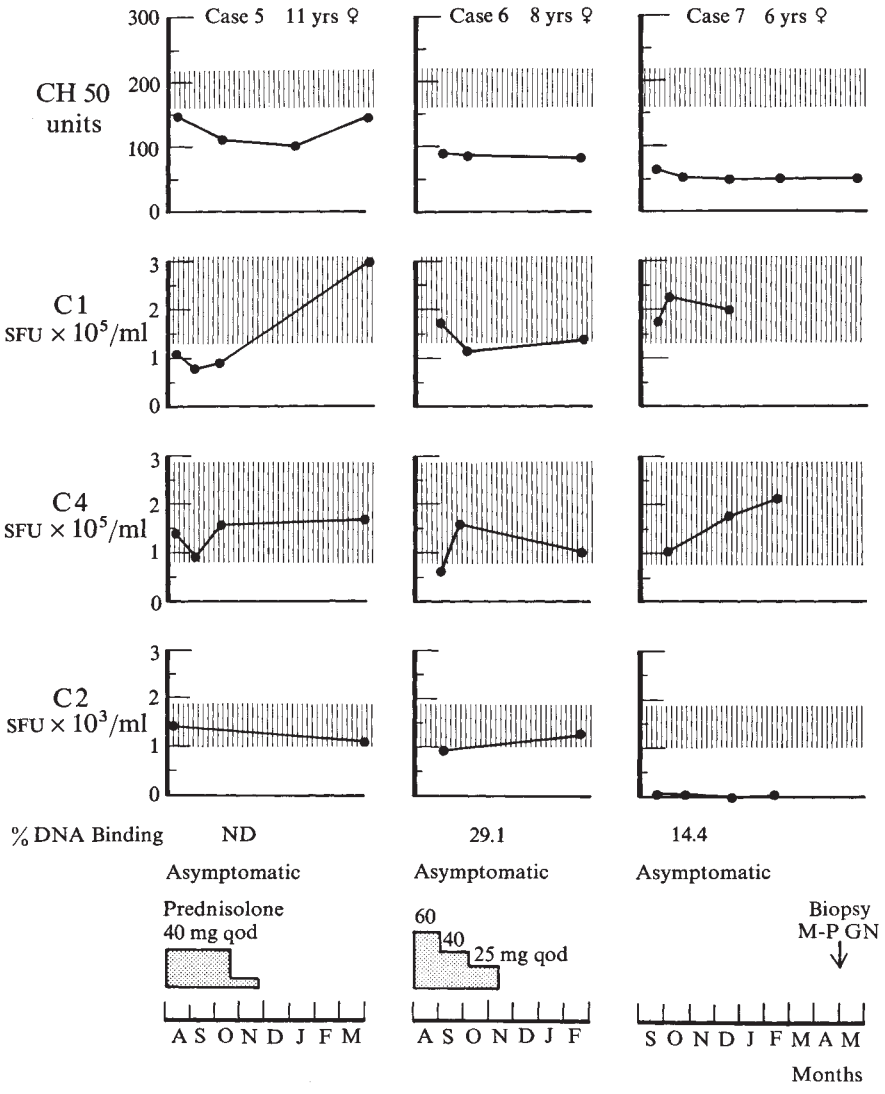

Fig. 2. Hemolytic titrations of whole complement (CH_{50}), C1, C4 and C2 in three patients with hypocomplementemic nephritis. The shaded zones indicate the range of C values in control sera.

Effect of immune complexes on component protein concentration. The results of incubating immune complexes with normal serum, shown in Table 4, demonstrated that 70% of the C1q, 57% of the C1s, 15% of the C4, and 25% of the C3 initial protein concentration was removed by this treatment. On the other hand, C1-inhibitor protein concentration remained unchanged. This led to an increased C1-inhibitor/C1 ratio in the supernatant as a consequence of complement activation by immune complexes.

Anti-DNA antibodies. The percent of DNA-binding by the Farr technique [20] was elevated in active systemic lupus erythematosus, especially when complement titers were depressed, but it fell during immunosuppressive therapy or clinical remission (Fig. 1).

Histology. Renal biopsies of subjects with systemic lupus erythematosus showed glomerular proliferation and membrane thickening compatible with the diagnosis of systemic lupus erythematosus. The three patients with hypocomplementemic glomerulonephritis had membranoproliferative lesions as described previously [7], with lobular accentuation, mesangial stalk thickening and hypercellularity, and basement membrane splitting. The changes were irregular in Case 6 and severe in Case 5. Fluorescent antibody staining was done on biopsies from three children. In systemic lupus erythematosus (Case 1), IgG and C3 were deposited on the glomerular basement membrane in a typical granular pattern. In hypocomplementemic glomerulonephritis, both biopsy specimens

were positive for granular deposits of C3 on the glomerular basement membrane. Case 5 showed a positive glomerular stain for IgG and fibrinogen. Case 7 exhibited quantitatively little staining for IgG and it was negative for IgM, IgA and fibrinogen. Properdin was demonstrated diffusely in a granular pattern on the glomerular basement membrane and mesangium.

The authors have not had extensive experience with the anti-properdin reagent – the antiserum was identical to that used in immunofluorescent studies of renal glomeruli by Westberg et al [29].

Table 3

C1 hemolytic activity

Case No.	Date	C1, Specific activity		Ratio C1 Inh/C1s
		C1/C1q	C1/C1s	
		Systemic lupus erythematosus		
1	11-6-70	44	36	2.44
	2-8-71	475	770	1.20
	5-20-71	909	404	1.20
2	11-9-70	435	830	3.10
	2-11-71	700	2500	3.40
	5-6-71	614	980	1.55
3	10-1-70	1450	1000	3.10
	1-19-71	1330	1800	1.74
4	2-23-71	1080	2000	1.60
	4-27-71	970	1950	1.70
	Normal	1000–1400	1500–2000	0.7–1.20
		Hypocomplementemic glomerulonephritis		
5	9-3-70	533	930	1.00
	4-1-70	1270	1760	
6	9-3-70	1306	1416	0.71
	2-16-71	1000	2000	
7	9-3-70	770	1100	0.90
	12-17-70	784	1280	
	Normal	10000–1400	1500–2000	0.7–1.20

[a] Specific activity = hemolytic units/immunochemical concentration of C1q or C1s.

Discussion

The present observations, which confirm previously reported differences in the complement profiles of patients with systemic lupus erythematosus and hypocomplementemic glomerulonephritis, are based on *in vitro* studies of the complement components in sera. Such data must be viewed in the light of several variables: different rates of synthesis, existence of functionally aberrant molecules, presence of inhibitors and variable pathways of activation (including immunological reactions and the action of enzymes generated by the clotting system or released from lysosomes). Theoretically, the sparing of early components in hypocomplementemic glomerulonephritis might result from efficient usage of these components via the conventional pathway or from a different mechanism of complement activation. Immunofluorescent studies of kidney biopsies showing granular deposits IgG, C1q and C3 on the glomerular basement membrane are compatible with efficient utilization of early components subsequent to immune complex activation. However a number of studies have demonstrated that C3 and the terminal components can be activated by lipopolysaccharides [21], zymosan [5], cobra venom [22] and by pseudoglobulins in the serum of patients with hypocomplementemic glomerulonephritis [23, 24, 25]. Recently an alternate pathway for the activation of C3–9 has been described involving the activation of C3-proactivator from its inactive precursor

Table 4

Depletion of complement components of normal human serum after incubation with BSA-anti-BSA complexes[a]

Component	C1q *μg/ml*	C1s % Normal	C4 *μg/ml*	C3 *mg/ml*	C1-Inh % Normal
Initial Concentration	150	72.5	384	1.22	75.8
Final Concentration	45	31.2	286	0.925	75.8

[a] One ml of normal human serum was added to washed preformed BSA-anti-BSA complexes (1.0 mg antibody protein) at equivalence, incubated for one hour at 37° C and centrifuged. Protein concentration of each subunit or component was assayed by radial immunodiffusion of the supernatant.

state [9, 26]. Serial studies of complement components in our patients with hypocomplementemic glomerulonephritis do not suggest immune complex activation, but instead point to C3 activation by this alternate pathway.

Suppression of antibody response in an immune complex disease by immunosuppressive drug therapy should be reflected by improvement in clinical status and relevant laboratory tests. The rise of complement components to normal in patients with systemic lupus erythematosus who were treated with adequate dosages of steroids or with cyclophosphamide suggests that drug therapy depressed immune complex formation in this disease. Further support for the suppression of immune complex formation is found in the tendency for elevated anti-DNA titers to fall concurrent with the rise in complement titers. In contrast to systemic lupus erythematosus, the failure of CH_{50} and C3 levels to rise significantly in patients with hypocomplementemic glomerulonephritis after courses of intensive immunosuppressive therapy suggests that immune complex formation is not a factor in the pathogenesis of this disease.

The decreased specific activity of C1 (C1/C1q and C1/C1s ratios) in patients with lupus erythematosus may be influenced by C1-inhibitor. The concentration of inhibitor, although increased in some sera (Table 2), was not consistently elevated or depressed, but the concentration of inhibitor relative to the concentration of C1 was increased (Table 3). A similar increase of the C1-inhibitor/C1 ratio has been described in rheumatoid synovial fluids with low CH_{50} levels [27]. An *in vitro* model, in which BSA-anti-BSA complexes are incubated with normal serum, showed depletion of C1q, C1s, C4 and C3 without decrease in inhibitor protein concentration. It is proposed that the increased C1-inhibitor/C1 ratio in the serum of children with systemic lupus erythematosus is an indication of immune complex activation and the consumption of C1. The absolute concentrations of C1 and C1-inhibitor, and the ratio of C1-inhibitor/C1, were normal in children with hypocomplementemic glomerulonephritis, thus supporting the thesis that the alternate pathway is activated in this syndrome.

The complement profile with selective lowering of C2 and C3 in a child with hypocomplementemic glomerulonephritis has not been described previously. Possible explanations for depressed C2 include: decreased synthesis or the presence of a specific inhibitor or inactivator. Hereditary C2-deficiency unassociated with disease [10] and nonfamilial C2-deficiency in a child with membranous glomerulonephritis [28] have both been documented. In order to determine whether our patient represented an example of hereditary C2-deficiency who inadvertently developed hypocomplementemic glomerulonephritis, C2 titers were done on all available family members, 6 siblings and the mother. Since hereditary C2-deficiency is an autosomal recessive trait and heterozygous individuals have moderately depressed C2 levels, the finding of a normal C2 level in the mother was not consistent with this form of deficiency. In view of the failure of the C2 and C3-deficient serum of our patient to inactivate C2 or C3 in normal serum, the presence of an inhibitor or inactivator seems unlikely. In fact depression of C2 to less than one-third of normal (390 SFU) in a healthy brother of the proband does suggest a congenital basis other than the previously described hereditary pattern of deficiency. Theoretically, the congenital absence of C2 could block the activation of C3 by the conventional pathway and provide evidence for the alternate pathway of C3 activation in hypocomplementemic glomerulonephritis. Immune adherence, a function of C3b, is normal even in the presence of marked C2-deficiency [14]. In this setting, minimal quantities of C2 may activate C3 through C3-convertase in the classical pathway, or C3b may be generated by the activation of C3-proactivator in the alternate pathway.

The child described previously with glomerulonephritis and C2-deficiency had membranous glomerulonephritis, a definitive pathologic entity, and normal serum C3 [28]. However, the development of glomerulonephritis, albeit of different types, in two C2-deficient children, raises the interesting query: do congenital complement abnormalities predispose to the development of glomerular disease?

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